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Reproductive Cycling in Female *Fundulus heteroclitus*

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Abstract. The ovaries of female *Fundulus heteroclitus* living in the northeastern Florida saltmarsh recrudesce in January and the fish initially spawn heavily during the subsequent full moons (a lunar pattern); they later spawn with less intensity during both the new and full moons (a semilunar pattern), and then regress in late September. In the laboratory, fish spawning against a vertical screen showed only semilunar periodicities, as observed for seven spawning groups under constant conditions (temperature 26 ± 1°C; photoperiod 14 h light to 10 h dark; excess food). Regardless of collection times (January, April, August, or September), these seven groups exhibited similar patterns of semilunar spawning for five to eight consecutive cycles whose periods (14.4 to 16.0 days) and phases (−1.7 to +8.4 days) were variable compared with concurrent full/new moon and spring tide cycles. These semilunar cycles, which occurred over the entire year in the laboratory, were thus free-running without entrainment and represent endogenous circasemilunar rhythms. In addition to annual and lunar/semilunar cycles, a tidal spawning cycle was also observed in the habitat. Fish apparently select the higher of the two semidiurnal tides for spawning, regardless of the daily light-dark cycle. This tidal cycle has not yet been tested in the laboratory.

Introduction

Most reproductively competent female fish exhibit annual cycles of ovarian development (Lam, 1983; Bye, 1984). The cycle begins with ovarian recrudescence, continues through a breeding period, and ends with ovarian regression. The recrudescence (which primarily involves vitellogenesis) is mostly under the influences of seasonal temperature and photoperiod. Later, during the breeding period, one or more clutches of ovarian follicles may develop progressively (Wallace and Selman, 1981a), often in synchrony with a combination of transient changes in temperature, illumination, spawning substrate availability, rainfall, moon phase, and tide, etc. (Lam, 1983; Bye, 1984; Stacey, 1984; Taylor, 1984). Ultimately, mature follicles ovulate and fish spawn at a specific time, a particular site, or both to maximize the immediate survival of eggs and hatchlings (Stacey, 1984; Taylor, 1984). Afterwards, a reproductive regression sets in to ensure that no hatchlings emerge and subsequently face a harsh environment inimical to their survival: follicles cease their growth and the ovary remains regressed until the next seasonal recrudescence. The seemingly direct responses of reproductive cycles to environmental influences do not necessarily exclude the involvement of endogenous organismal rhythms (Lam, 1983; Bye, 1984; Stacey, 1984). Such self-sustained rhythms schedule reproductive preparation, time receptivities for external cues, and, most important of all, can be entrained to follow repetitive environmental parallels (Elliot and Goldman, 1982).

Definitive studies of these reproductive cycles require a combination of both field observations and laboratory testing. However, the obvious complexity of reproductive physiology and its correlation to surmised environmental cues pose a difficulty for laboratory research. It is not surprising that most of the studies to date have been conducted in the field, but recent laboratory progress is encouraging. The endogenous nature of the annual cycle and the environmental cues that can phase-shift the cycle have been revealed in long-term laboratory observations (Bye, 1984; Bromage *et al.*, 1990), as exemplified by studies on rainbow trout, *Salmo gairdneri* (Scott and Sumpter, 1983; Duston and Bromage, 1991), stickleback, *Gasterosteus aculeatus* (Baggerman, 1980), and catfish, *Heteropneustes fossilis* (Sundararaj *et al.*, 1982). In these fish, ovarian development beyond recrudescence has been as-

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sumed to proceed spontaneously under the sole regulation of a circannual timer. However, the involvement of additional timers has never been excluded. For that matter, specific timers for follicle maturation and ovulation have been identified in other fish. For example, in the medaka, *Oryzias latipes*, a circadian timer apparently schedules the daily ovarian maturation for spawning at light onset (Iwamatsu, 1978; Ueda and Oishi, 1982; Weber and Spieler, 1987), and in the Gulf killifish, *Fundulus grandis*, a temperature-compensated circasemilunar timer is involved in ovarian maturation for spawning during spring tides (Hsiao and Meier, 1988, 1992). Although the circadian timer in medaka further regulates the timing of ovulation and spawning, in goldfish, *Carassius auratus*, the two processes, ovarian maturation and ovulation and spawning, are separately regulated. A gravid female goldfish with fully grown follicles does not spawn until warm temperature and a suitable spawning substrate become available; it then ovulates, under a circadian control, at midnight and spawns at first light (Stacey *et al.*, 1979).

Of all the fish employed in the above-mentioned studies, only *F. grandis* spawns with an important lunar/semilunar pattern related to semilunar tidal or moonlight cycles. Through the breeding season, this species and many other cyprinodonts exhibit lunar or semilunar spawning cycles that coincide with spring tides associated with synodic moonlight (29.6 days) on the Atlantic coast or with lunar declinational cycles (27.3 days) on the Gulf coast (Taylor, 1984). Depending on available habitat, they deposit their eggs during the daily high tides into empty mussel shells, marsh grass, or sand near or on the flooded surface of the marsh at or above the mean high waterline (Taylor, 1984). When the high tides recede, the eggs are left to incubate in a protected and humid environment until subsequent spring tides again flood the spawning site and the eggs hatch. One apparent advantage to such a reproductive strategy is that it ensures a predator-free and oxygen-rich location for developing embryos (Taylor and DiMichele, 1983). This strategy, however, demands an extraordinary organismal coordination with tidal/lunar environments.

Laboratory examination of semilunar/lunar reproductive activities in cyprinodonts has been initiated only recently (Taylor, 1986; Hsiao and Meier, 1986). Gradually, *Fundulus heteroclitus* has emerged as a promising laboratory model (Hsiao and Meier, 1989; Lin *et al.*, 1989; Taylor, 1991). Although this fish has been used in physiological, embryological, genetic, and environmental studies for decades (Huver, 1973; Atz, 1986), only in the past few years has it been shown to exhibit annual, lunar, semilunar, and tidal spawning cycles in the habitat (Day and Taylor, 1984; Taylor, 1984, 1986) and semilunar cycles in the laboratory (Hsiao and Meier, 1989; Taylor, 1991). Attention has recently been given to the interplay among internal hormonal rhythms, endogenous rhythmicity, and external entraining cues (Bradford and Taylor, 1987; Cochrane *et al.*, 1988; Hsiao and Meier, 1989; Taylor, 1991). Developmental aspects of the reproductive cycles, particularly the relationship between follicle growth and cyclic spawning, have also been examined (Taylor and DiMichele, 1980).

In the present investigation, the field and laboratory observations on reproductive cycling in female *F. heteroclitus* were extended at a northeast Florida site. Fish were sampled at intervals of about 2 days during a breeding season from January through October. Fish were also moved from their habitat into the laboratory several times during the year and their spawning patterns observed. Comparisons between field and laboratory data reveal mechanisms underlying the annual and semilunar reproductive cycles and indicate further research possibilities.

Materials and Methods

Field studies

Fish. Specimens of *F. heteroclitus* living in the saltmarsh along the Intracoastal Waterway near the Whitney Laboratory (St. Augustine, 29°40′ N latitude and 81°13′ W longitude) were collected with unbaited minnow traps. In 1984, samples containing five medium or large females [46 mm SL and larger, as classified by Kneib (1986)] were collected during daytime incoming tides at intervals varying from 1 to 4 days from early January through mid-September. Afterward, sampling was continued less frequently through mid-October. During spawning episodes in March and May 1985, females were also collected and examined at 3- to 4-h intervals over two consecutive spawning days to observe ovarian preparations for immediate spawning.

Adjusted tide tables (*Reed's Nautical Almanac & Coast Pilot*) were used to predict spring tides and semidiurnal high tides at the collection sites. In addition, tide measurements were taken by a mechanical recorder at the Whitney Laboratory dock on the Intracoastal Waterway to accurately predict daily high tides.

Fish were anesthetized with a solution of 3-aminobenzoic acid ethyl ester (MS-222; Sigma) within 1 h after capture. The fish were then weighed to the nearest 0.1 g and sacrificed by cervical incision. The single ovary (a fusion of two lobes) of each fish was removed, blotted dry, and weighed to the nearest milligram. A gonadal-somatic index (GSI = gonad weight × 100/total body weight) was then calculated.

Ovaries containing ovulated eggs were often difficult to handle without losing the eggs. To minimize error, we flushed ovaries before weighing: each ovary was opened laterally with sharp scissors and its eggs were shaken into a petri dish and counted as total eggs for a particular fish.

The final weight of each ovary was amended by the addition of egg weight estimated from the egg number and the average weight of a single egg [3.7 mg (Greeley *et al.*, 1991)].

Follicular development. Follicular development was indicated by stages and size distribution of follicles in each ovary. A small wedge-shaped sample of fresh ovary (minus ovulated eggs) was carefully cut with sharp scissors from the midportion of an ovarian lobe. Each sample was then placed individually in a petri dish containing a culture medium, FO solution (Wallace and Selman, 1978). Remaining ovarian tissue was reweighed to determine the proportion of the total ovary represented by the sample. Follicles in the sample were teased apart and those with diameters equal to or larger than 0.5 mm were measured to the nearest 0.1 mm (generally about 200 follicles per ovary piece). The apparent developmental stage of each follicle size-class was categorized according to morphological criteria (Selman and Wallace, 1986), and the presence and quantity of atretic follicles were noted (atretic follicles appeared somewhat opaque with irregular and flattened surfaces). Because follicle development is essentially uniform throughout the *F. heteroclitus* ovary (Taylor and DiMichele, 1980), measurements from each sample were proportionally converted to the total number of eggs or follicles per ovary. Finally, the data were standardized to a total egg or follicle number per gram of total body weight.

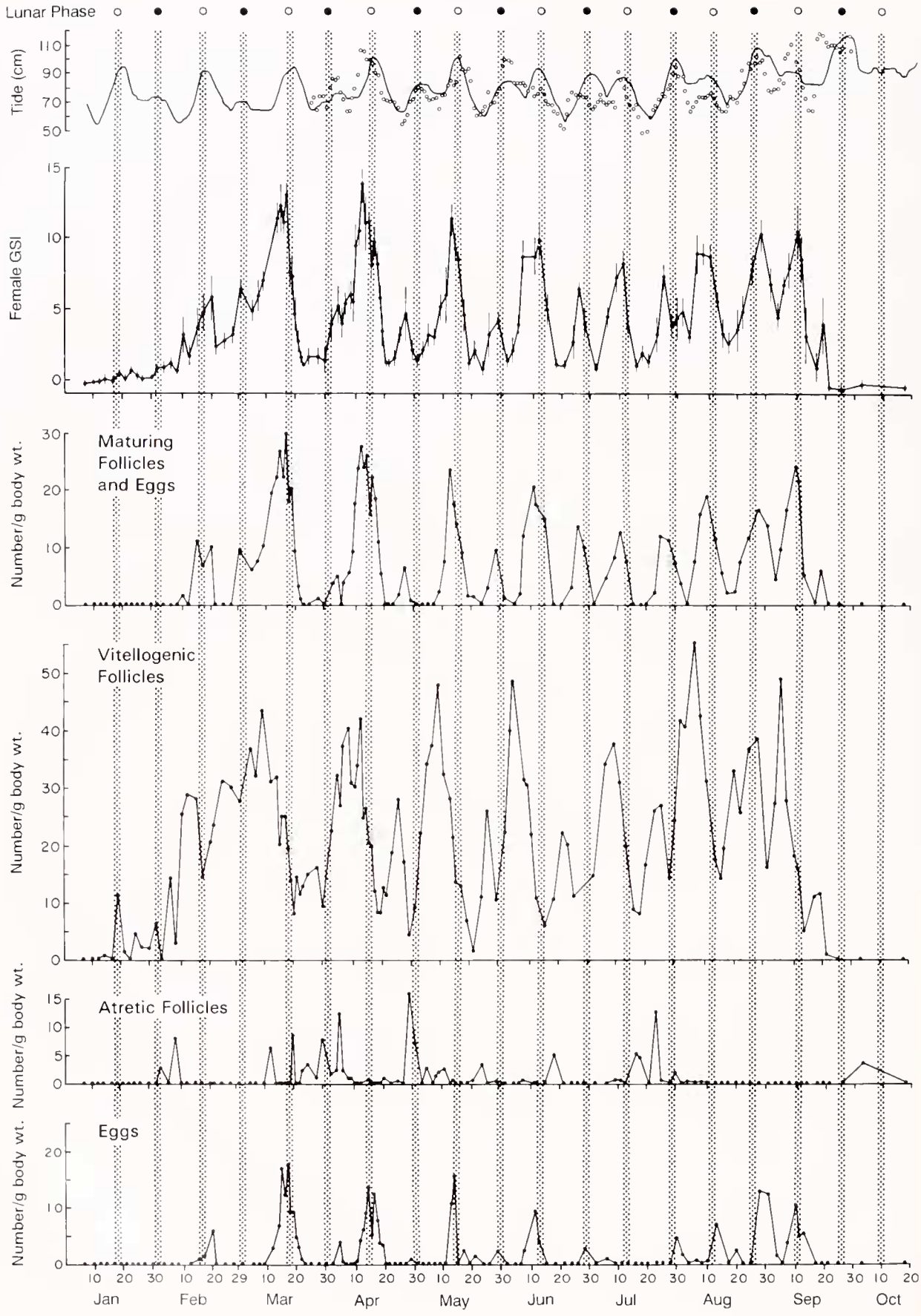
Data analysis. Due to the lack of a consistent daily or hourly continuity, the field data collected over months and over days were not analyzed through spectral time-series calculations. Instead, cycles were displayed graphically. In addition, the ovarian cycles were graphically compared with concurrent environmental cycles of high tide, moonlight, and daily photoperiod (Figs. 1–4).

Laboratory studies

Fish and spawning observations. Specimens of *F. heteroclitus* living in the saltmarsh surrounding the Intracoastal Waterway near the Whitney Laboratory were also collected for laboratory studies. Large fish—56 mm SL and larger, as classified by Kneib (1986)—were collected in January, April, August, and September 1991. Unbaited minnow traps were placed in the saltmarsh for as long as 24 h to obtain a large quantity of fish in one collection. In the laboratory, fish were kept in 550-l plain tanks or grouped in 550-l, 66-l, or 10-l partitioned spawning tanks. The concentrations were 188–300 fish per 550-l tank, 27 fish per 66-l tank, and 19 fish per 10-l tank. Each spawning tank was partitioned by a vertical black plastic screen with quarter-inch mesh to provide a narrow isolated compartment. Fish tend to spawn against the screen and eggs fall into the protected compartment. All tanks were aerated and filled with flowing seawater at 26.5 ± 1.5°C and 35 ppt salinity throughout the year. The lighting was fluorescent with a photoperiod of 14 h light to 10 h dark (LD 14:10). Twice daily, fish were fed to satiety with flakes (Wardley) that were left in the tank for at least 10 min after the initial vigorous feeding. The flakes were supplemented with cooked egg and crushed shrimp twice a month.

In each spawning tank, a fish group with a sex ratio of one male to three to eight females was maintained. The spawning was monitored by siphoning eggs out of the isolated compartment every 24 h. This technique was developed in studies of *F. grandis* and *F. heteroclitus* conducted in 38-l aquaria (Hsiao and Meier, 1986, 1989). To test whether our 550-l spawning tanks required extra spawning sites, we equipped the 550-l tank containing our first fish group A (250 females and 50 males) with an additional plastic screen folded as a cone and suspended in the middle of the tank. The cone was 30-cm long with a pointed top and an open bottom connected to a removable cup. Through daily removal of eggs from the cup, we monitored spawning at this extra site, which was found to be unnecessary (see Results); the remaining spawning tanks were equipped with vertical screens only.

Data analysis. Daily counts of eggs collected from the various fish groups were analyzed through SPECTRA time-series calculations (SAS Institute, Cary, North Carolina) to detect the presence of repeating cycles and their approximate periods. Nonlinear regression was used to fit sine curves to data series in which cycles had been identified. For each data series, ranges of sine curve amplitudes, phases, periods, and means were tested in extensive trials to produce the best fit for all or part of the series. Afterward, the fitted portion of a data series was divided into individual cycles at the lowest points of the matching sine curve with the original data bracketed in the cycles. A mean spawning date (±95% confidence limits) in each cycle was then calculated. This calculation often gave a peak spawning date slightly different from the date marked by the fitting sine curve. The mean spawning date was subtracted from the nearest new or full moon, thus providing the phase relation between the actual spawning peak and the concurrent moonlight cycle. Negative and positive phases respectively indicate that the spawning peak occurred before and after the new/full moon (Table I). The phases of successive cycles were analyzed by linear correlations to see if the variable spawning cycles of a data series have advanced or delayed sequentially in relation to the constant concurrent moonlight cycles (Table I). The phase relations were also graphed with parallel displays of daily egg counts for the various


Lunar Phase
Tide (cm)
Female GSI
Maturing Follicles and Eggs
Vitellogenic Follicles
Atretic Follicles
Eggs
Number/g body wt.
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct

fish groups, concurrent cycles of moonlight, and predicted local tides (Figs. 5, 6).

Results

Field studies

Annual reproductive cycle. An annual reproductive cycle, containing lunar and semilunar spawning cycles, of females of *F. heteroclitus* inhabiting the northeastern Florida saltmarsh is illustrated by their variations in GSI and ovarian profile (Fig. 1). In January and early February, vitellogenic follicles gradually increased to the baseline of their cyclic appearance from February to September. In this later period, each cycle of vitellogenic follicles grew into a cycle of maturing follicles and ovulated eggs which, because of their relatively large size, contributed to high GSIs. The coexistence of high GSIs and maturing follicles plus eggs was transient, indicating a periodic discharge of ovarian products due to spawning. Thus, the first spawning episode in 1984 was identified to be during the spring tides associated with the full moon in mid-February. Subsequently, spawning episodes appeared successively at approximately lunar (29.6-day) or semilunar (14.8-day) intervals throughout the lengthy breeding season extending through September.

During the breeding season, in addition to exhibiting cyclic support for the spawning cycles, ovaries also revealed a discrete change in the size-frequency distribution of vitellogenic and maturing follicles (Fig. 2). At early successive full moon spring tides (*i.e.*, 15 February, 15 March, and 12 April), clutches of these follicles could not be easily distinguished from one another by size alone. As the season advanced, however, follicle clutches became more distinct. Later, during successive full moon spring tides (*i.e.*, 9 July, 10 August, and 11 September), clutches of follicles, particularly maturing follicles, rarely overlapped with smaller vitellogenic follicles. The size of the largest maturing follicles also tended to decrease during the breeding season. The formation of follicle clutches did not continue into the fall when the production of vitellogenic follicles suddenly ceased, resulting in a decrease in both maturing follicles and ovulated eggs and a subsequent increase in atretic follicles (Fig. 1). At this point, the breeding season ended.

Lunar and semilunar spawning cycles. After the first GSI peak at mid-February full moon, the next GSI peak occurred at the mid-March full moon and was followed by another GSI peak at the April full moon (Fig. 1). Between these early major GSI peaks were two minor GSI peaks associated with the new moon spring tides, but these minor peaks appeared to merge with the following full moon major peaks. Thus, there were three distinct large lunar spawning cycles during the early breeding season. It was not until late April that a well-defined new moon GSI peak appeared. Through time, size of the new moon GSI peaks increased while that of the full moon GSI peaks decreased until they were approximately equal after late June. Most GSI peaks began their decline right before or at new moon or full moon in coincidence with spring tides, indicating that spawning (discharge of ovarian products) strictly occurred at spring tides. The coincidence was lost in September; the last GSI peak, a minor one, occurred between the full and the following new moon. Overall, the breeding season was initially characterized by a lunar pattern of heavy spawning and later by a semilunar pattern of lighter spawning (Fig. 1). In addition, all lunar/semilunar spawning except the last cycle was consistently in synchrony with new/full moon cycles and the corresponding spring tides.

Throughout the study, the total number of maturing follicles and eggs (reflecting ovarian products that will soon be spawned) corresponded well with the GSI values (Fig. 1). To a lesser extent, this was also true for vitellogenic follicles, although their numbers tended to peak a few days earlier than those of maturing follicles plus eggs. Taken alone, however, the number of eggs in the ovary corresponded poorly with the cyclic fluctuation of GSI values and was not a good indication of spawning activity. This was probably because animals were collected only during the day, so females that spawned during the early morning hours were devoid of eggs when captured, unlike females that spawned in the late afternoon or early evening (see below).

Follicular growth cycles. A series of ovarian activities underlay each lunar or semilunar spawning cycle: vitellogenic follicles grew and accumulated and then passed on to form a peak of maturing follicles and ovulated eggs

Figure 1. Variations in gonadal-somatic index, (GSI, mean ± SD), the combined count of maturing follicles and eggs, vitellogenic follicles, atretic follicles, and ovulated eggs in *Fundulus heteroclitus* females collected from a northeastern Florida saltmarsh over the course of a breeding season. Each point represents a value derived from five fish. The predicted maximum height of daily high tide appears as a solid curve near the top of the figure, and the actual measurements of the maximum daily high tide are represented as small unfilled circles (discrepancies are due primarily to marsh structures and prevailing winds). Lunar phases are indicated at the top as new moons (large filled circles) and full moons (large unfilled circles). Vertical dotted bars mark the dates of new and full moons.

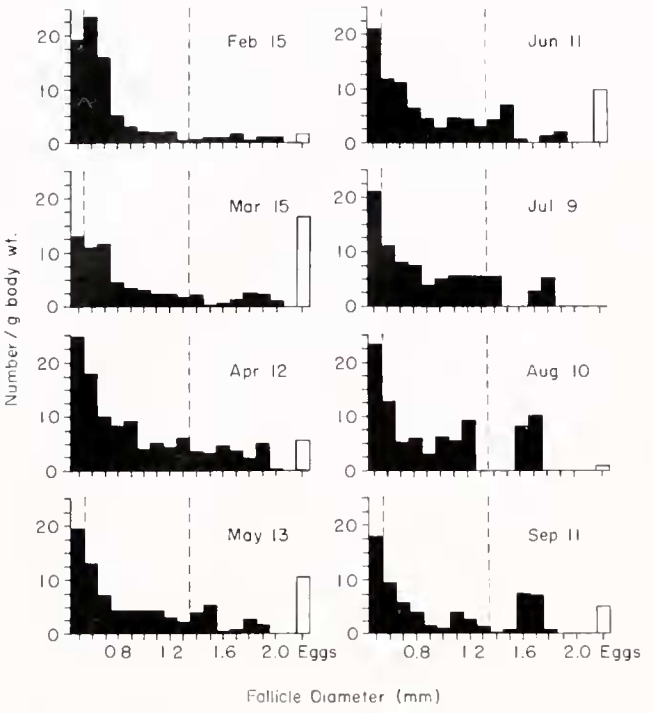


Figure 2. Subtle variations in the ovarian profiles (follicle size/frequency distributions) of *Fundulus heteroclitus* females collected during full moon spring tides in successive months of a breeding season. Each profile provides the mean number of various size follicles (filled bars) and ovulated eggs (unfilled bars) found for five fish on each sample date. In each histogram, the left dashed vertical line marks the transition from the cortical alveoli stage of development into vitellogenesis, and the right dashed vertical line marks the transition from vitellogenesis into maturation, as defined by Selman and Wallace (1986). Note the increased formation of distinct follicle clutches as the months progressed.

(Fig. 1). Any failures in this process would result in atretic follicles, but these did not occur regularly during our observations (Fig. 1). Details of the vitellogenesis-maturation-ovulation transition during a lunar cycle were revealed by the ovarian profiles collected at 2-day intervals over a 23-day period (Fig. 3). After a minor new-moon spawn (29 April), the ovaries examined had few early vitellogenic follicles, no maturing follicles, and only a few ovulated eggs. During the next several days, however, mid- to late-vitellogenic follicles appeared and then accumulated in the ovary, and after the quarter moon on 7 May, a distinct clutch of maturing follicles also appeared. Afterward, vitellogenic follicles entered maturation, resulting in maturing follicles and ovulated eggs during days of spring tides (*i.e.*, 11, 13, 15 and 17 May) that occurred on and immediately preceded and followed the full moon (15 May). In these spawning days, follicles appeared to mature and ovulate in waves, one clutch after the other. At times (*e.g.*, 13 May), as one clutch began to mature, another was in late maturation, and the leading clutch had ovulated. This vigorous egg production exhausted the

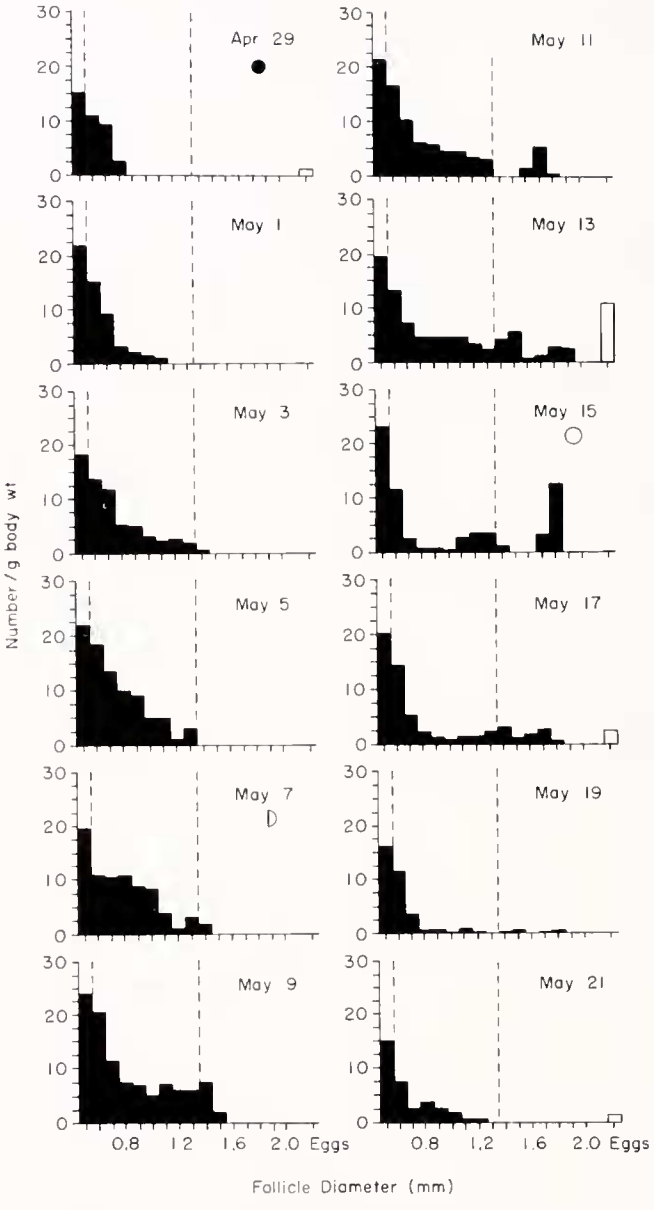


Figure 3. Variations in the ovarian profiles of *Fundulus heteroclitus* females collected during a 23-day interval encompassing a semilunar spawning period associated with the full moon (15 May). Each profile provides the mean number of various size follicles (filled bars) and ovulated eggs (unfilled bars) found for five fish on each sample date. In each histogram, the left dashed vertical line marks the transition from the cortical alveoli stage of development into vitellogenesis, and the right dashed vertical line marks the transition from vitellogenesis into maturation, as defined by Selman and Wallace (1986). Note the gradual accumulation of vitellogenic follicles, followed by recruitment of follicle clutches into maturation in preparation for spawning, and finally depletion of most larger follicles from the ovary. Lunar phases are indicated as new moon (filled circle), quarter moon (half unfilled circle), and full moon (unfilled circle).

pool of vitellogenic follicles by the end of the spring tide series on 19 May. Two days later (21 May), mid-vitellogenic follicles appeared again, thus initiating another cycle

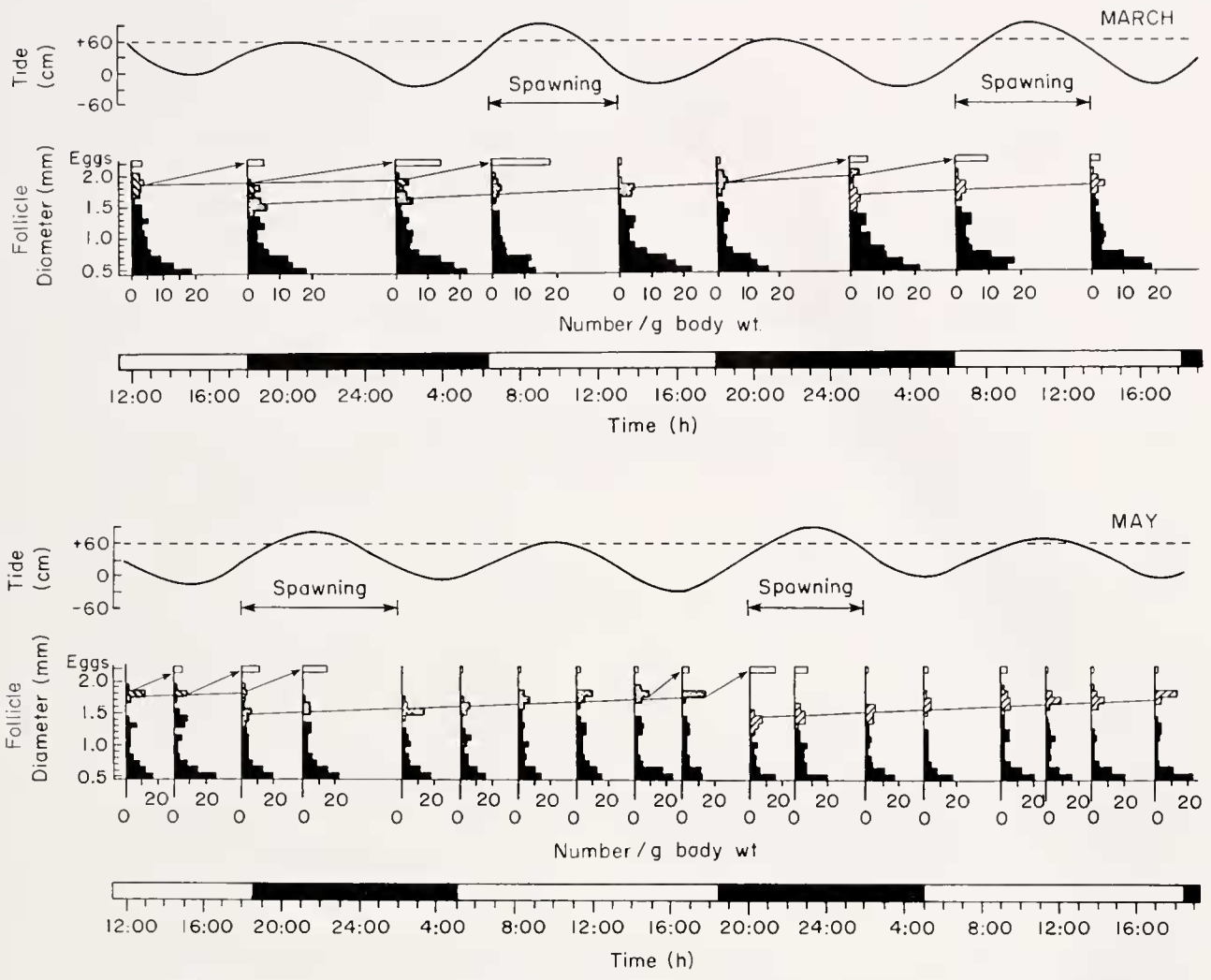


Figure 4. Ovarian profiles of *Fundulus heteroclitus* individuals collected over two-day periods of spring tides indicating preparations for selective tidal spawning. Samples were collected in either March or May (with time and photoperiod marked). Data for each time point represent the numerical averages for five (March) or six (May) females. Solid lines trace the successive development of three discernible follicle clutches during each two-day period. Arrows branching out from lines indicate the occurrence of ovulation. Semidiurnal tides at the time are presented as actual recorded tidal fluctuations from the mean water-line. Dashed lines through the tides reveal the approximate surface level of the upper saltmarsh.

of ovarian preparation for the next spawning episode. During this 23-day observation period, the numbers of late cortical alveolus-stage follicles (0.5-mm diameter) and early vitellogenic follicles (0.6- and 0.7-mm diameter) in each ovary remained relatively constant (Fig. 3). Apparently, the recruitment of follicle clutches in the ovary occurs instantly in early vitellogenesis, thus providing constant support for lunar/semilunar spawning by the repetitive and timely production of fully vitellogenic and maturational follicles.

Tidal ovulation and spawning. During days with spring tides, the ovary proceeds to its final preparation for spawning. The environmental correlates for the final steps are apparently not the semilunar moon phase or the semilunar tidal change, but the local semidiurnal tides. An apparent tidal spawning was observed immediately preceding the full moon of March and May 1985, with ovaries collected and examined at 3- to 4-h intervals over two consecutive spawning days in each month (Fig. 4). During the two days in March, a clutch of maturing follicles began to ovulate about 16-20 h before the spawning high tide. Ovulated eggs then accumulated and were spawned during the daytime high tide (note the disappearance of eggs from the ovary after the high tide). During the next 24 h, additional eggs were ovulated from another clutch of maturing follicles,

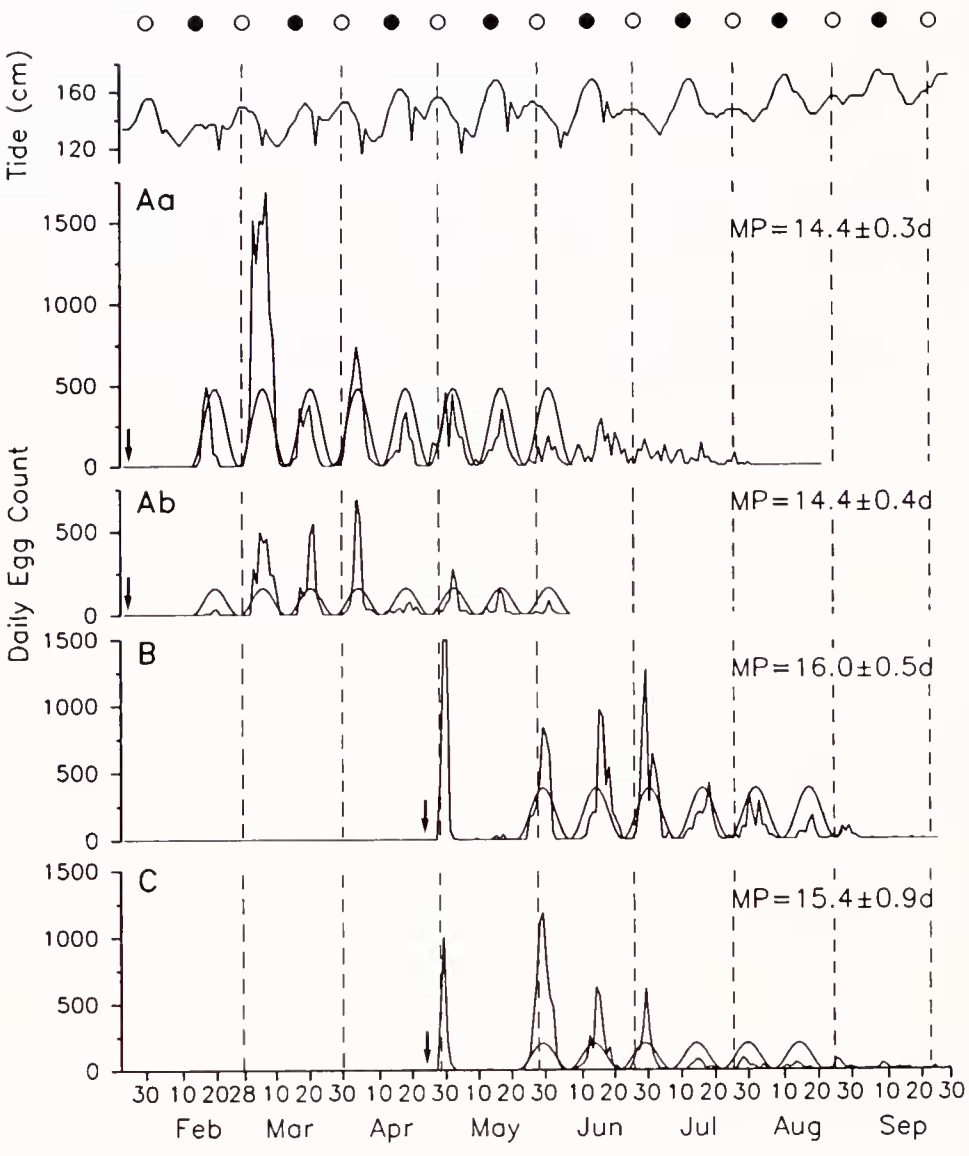


Figure 5. Cyclic variations in daily egg counts of fish groups A, B, and C. These groups were respectively obtained on 23 January, 24 April, and 26 April (marked by arrows). Spawning tanks holding each group were equipped with vertical screens as spawning substrates, with the exception of the tank for group A, which had a suspended screen cone as an extra substrate. Eggs collected from group A at the vertical and suspended screens are indicated as Aa and Ab, respectively. A part of each data series is significantly ($P < 0.01$) fitted by sine curves through nonlinear regressions with a calculated mean cycle period (MP) ± 95% confidence limits provided. Concurrent cycles of moonlight (full moon: unfilled circle; new moon: filled circle) and the predicted maximal daily high tide are indicated at the top of the figure for comparison. Vertical dotted lines mark the dates of full moons.

and spawning again occurred at the following daytime high tide. During the two days in May, a similar pattern was observed, but spawning occurred at the nighttime high tide. In both the March and May observations, two or three developing follicle clutches could be discerned over each two-day period. However, spawning occurred only at the higher of the two daily semi-diurnal tides, regardless of its daytime or nighttime appearance.

Laboratory studies

Semilunar spawning. Because the field data revealed a distinct annual reproductive cycle, fish collected from the

habitat at different seasons were expected to display various reproductive patterns—if any—in the laboratory. Surprisingly, regardless of their collection time, they all exhibited a similar reproductive progression containing five to eight semilunar spawning cycles (Figs. 5, 6).

In the winter, fish group A (250 females and 50 males in a 550-l spawning tank) was collected on 23 January and began spawning three weeks later. The spawning continued from February through July, with equivalent semilunar cycles observed at two egg collection sites, the vertical screen (Aa) and the suspended screen cone (Ab) (Fig. 5). The mean periods (14.4 ± 0.3 days for Aa and 14.4 ± 0.4 days for Ab) and phases (Fig. 5 and Table I) of eight consecutive cycles were similar for eggs collected at both sites and could be significantly correlated ($P < 0.01$) to sine curves. Accordingly, we concluded that a single egg collection site was sufficient to reveal the cyclic spawning of a captive group in the 550-l tank, and we used the vertical screen as the only collection site for eggs from fish collected subsequently. Although the semilunar cycles revealed by egg collections of Aa and Ab appeared similar to concurrent moonlight and tidal cycles, the dissociation between them was revealed by the differences in cycle periods and phases. The shorter mean cycle periods of Aa and Ab (as compared to a 14.8-day semilunar cycle) resulted in sequential reductions in their phases ($r = -0.77$, $P < 0.05$ in Aa; $r = -0.88$, $P < 0.01$ in Ab) relative to concurrent moonlight cycles: the mean spawning peaks in successive cycles occurred first at six days and drifted to two days after respective new/full moons (Table I).

In the spring, fish groups B (210 females and 25 males in a 550-l spawning tank) and C (163 females and 25 males also in a 550-l spawning tank) were collected four and two days, respectively, before the full moon on 28 April. On 28 and 29 April, both groups simultaneously spawned heavily in their separate tanks (Fig. 5). Afterward, there was a four-week pause before groups B and C spawned again, on another full moon (28 May). The semilunar spawning then lasted until August, and each group exhibited at least five consecutive cycles significantly matched by sine curves ($P < 0.01$). The mean periods were 16.0 ± 0.5 days for B and 15.4 ± 0.9 days for C. The longer periods of groups B and C (compared to 14.8 days) resulted in sequential phase delay ($r = 0.93$, $P < 0.01$ in B; $r = 0.90$, $P < 0.01$ in C) relative to concurrent moonlight cycles (Table I). The mean spawning peaks in successive cycles drifted from 1 to 8 days in B and 1 to 4 days in C after respective new/full moons (Table I).

In the summer, fish groups D and E (each containing 15 females and 4 males in a 10-l spawning tank) were collected on 20 August. They began spawning in the laboratory about three weeks later and continued spawning from September to December (Fig. 6). Although they were in small tanks, both groups also exhibited distinct semilunar cycles significantly ($P < 0.01$) fitted by sine curves for seven cycles. The mean periods were 14.4 ± 0.4 days for D and 14.5 ± 0.3 days for E (Table I). These relatively short periods (compared to 14.8 days) resulted in sequential phase advance ($r = -0.79$, $P < 0.05$ in D; $r = -0.86$, $P < 0.05$ in E) relative to concurrent moonlight cycles. The phases of successive cycles in D first went up from 3.7 days to 6.4 days and then dropped to −1.7 days and 1.1 days for the last two cycles. The phase progression in E indicated a smoother advance with a drift from four to about two days (Table I).

In the fall, a batch of fish was collected on 20 and 21 September and kept in a 550-l stocking tank without a vertical screen. A month later, on 26 October, the batch was divided into two groups, F and G (each containing 20 females and 7 males in a 66-l spawning tank). Thereafter, these groups spawned actively for three months from November through January, with distinct semilunar cycles significantly ($P < 0.01$) fitted by sine curves (Fig. 6). The mean cycle periods were 15.1 ± 0.7 days in F and 14.9

Table I

Mean period (MP) of semilunar spawning in laboratory fish groups A to G (±95% confidence limits) and phase shift against concurrent moonlight cycles

		Phase shift (days)[a]							
		Cycle:							
Group	MP	1	2	3	4	5	6	7	r[b]
Fish collected on 23 January, 1991									
Aa	14.4 ± 0.3	5.6	2.9	3.9	3.4	3.0	2.1	2.6	−0.77*
Ab	14.4 ± 0.4	6.2	4.0	4.4	3.6	3.8	1.7	2.5	−0.88**
Fish collected on 24 (B) and 26 (C) April 1991									
B	16.0 ± 0.5	1.0	4.1	3.8	5.5	5.4	8.4		+0.93**
C	15.4 ± 0.9	0.9	2.5	2.7	3.9	3.7	3.8		+0.90**
Fish collected on 20 August 1991									
D	14.4 ± 0.4	3.7	6.4	3.3	4.7	1.5	−1.7	1.1	−0.79*
E	14.5 ± 0.3	4.0	3.6	4.0	2.4	2.5	1.3	2.2	−0.86*
Fish collected on 20 and 21 September 1991									
F	15.1 ± 0.7	2.4	2.3	0.5	2.6	3.9			+0.60[ns]
G	14.9 ± 0.5	1.9	3.6	1.1	1.9	4.0			+0.36[ns]

[a] In each defined cycle fitted by sine curves (Figs. 5, 6), the date of the mean spawning peak (egg collection peak) was calculated and its phase relation to the concurrent moonlight cycle was determined. Negative and positive numbers respectively indicate the days that the peak occurred before and after the nearest new/full moon.

[b] * $P < 0.05$; ** $P < 0.01$; [ns]not significant.

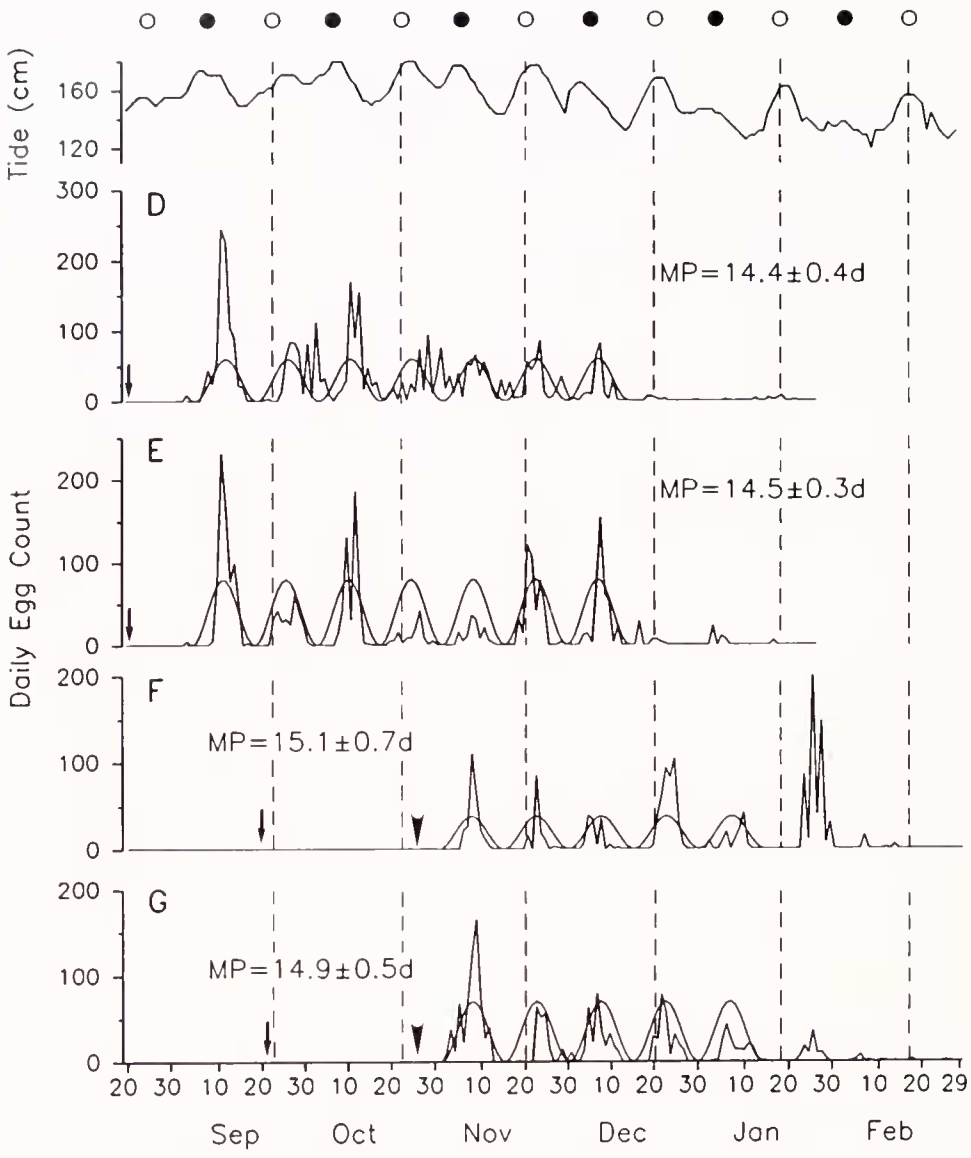


Figure 6. Cyclic variations in daily egg counts of spawning groups D, E, F, and G. Groups D and E and groups F and G were obtained from the habitat on 20 August and 20 and 21 September, respectively (marked by arrows). Groups F and G were initially held in a stocking tank for a month before being divided into two spawning groups on 26 October (arrowhead). A part of each data series is significantly ($P < 0.01$) matched by a sine curve through nonlinear regressions with a calculated mean cycle period (MP) ± 95% confidence limits provided. Concurrent cycles of moonlight (full moon: unfilled circle; new moon: filled circle) and the predicted maximal daily high tide are indicated at the top of the figure for comparison. Vertical dotted lines mark the dates of full moons.

± 0.5 days in G, with little difference from the 14.8-day moonlight cycle. Consequently, the spawning cycles stayed in phase and actually in synchrony with the concurrent moonlight cycle (Table I).

Overall, in addition to the indifference of laboratory spawning to season, the independence of semilunar spawning cycles from the concurrent moonlight cycle is apparent. Mean semilunar periods of the seven groups examined ranged from 14.4 to 16.0 days, and their cycle phases in relation to concurrent moonlight were not consistent. In contrast to the synchrony between lunar/semilunar GSI variations and moonlight cycles in wild fish (Fig. 1), there was no evidence that the laboratory spawning cycle was entrained or driven to follow a common cycle of moonlight or spring tide. Thus, the laboratory cycling was endogenously timed, free-running, and independent of environmental cues.

Furthermore, all seven laboratory groups, after about three to four months of spawning, appeared to enter a nonreproductive state, even though laboratory conditions (temperature, photoperiod, and feeding) remained the same. Although the spawning groups were monitored for one to two months after cyclic spawning ceased, the length and reversibility of the nonreproductive state were not further investigated.

Discussion

The extended field studies revealed the existence and interplay of annual, lunar/semilunar, and tidal reproductive activities. Parts of these activities persisted in the laboratory and are thus amenable to investigations of their underlying physiological mechanisms, particularly neurohormonal signaling along the hypothalamic-hypophyseal-ovarian axis. However, until the coexistence of annual, semilunar/lunar, and tidal reproductive activities can be more completely demonstrated in the laboratory, the interactive mechanisms among these cyclic activities cannot be well addressed.

Annual reproductive cycle

During our 10-month sampling period, females of *F. heteroclitus* inhabiting the northeastern Florida saltmarsh exhibited a distinct annual reproductive cycle. Their ovaries enlarged in late winter when the number of vitellogenic follicles gradually increased. During their breeding season in spring and summer, ovarian preparation for spawning occurred in cycles, with a peak of vitellogenic follicles preceding a peak of maturing follicles and ovulated eggs, leading to a spawning episode at spring tides (Fig. 1). From February to April, spawning peaks were large and took place at full moon spring tides, indicating a lunar pattern for the three cycles observed in 1984. From April to September, smaller spawning peaks occurred during both full and new moon spring tides, showing a semilunar pattern for the 10 cycles observed in 1984. Termination of the breeding season in late September was marked by a quick decline in vitellogenic and maturing follicles and a short period of irregular spawning. The fish then entered a reproductively quiescent phase. A similar annual cycle, including a breeding season during February through September with a transition from a lunar to a semilunar spawning pattern, has also been reported for *F. heteroclitus* in Georgia (Kneib, 1986). A less-detailed description of the annual cycle for *F. heteroclitus* on a north Carolina marsh reveals high GSIs from March through May and lower GSIs from June to August in reproductive females (Kneib and Stiven, 1978). The annual cycle for a Delaware population of *F. heteroclitus*, described in segments through several studies, has a restricted breeding season between April and August with a mixture of lunar and semilunar ovarian maturation cycles (Taylor *et al.*, 1979; Taylor and DiMichele, 1980; Day and Taylor, 1984; Taylor, 1986). The Massachusetts population living in the Woods Hole area has an even more restricted spawning season—from May through early summer—and the ovaries of these fish constantly contain maturational oocytes and eggs in May (Wallace and Selman, 1981b). It appears that this species, in general, spawns heavily when the temperature begins to rise and may extend its spawning opportunistically in the southern locations at summer high tides. Such an annual pattern was also observed in *Fundulus grandis* individuals living on the Alabama Gulf coast (Greeley and MacGregor, 1983; Greeley *et al.*, 1988). By exerting greater reproductive effort at the beginning of the season, a species may maximize its offspring's survival by ensuring a long growing season for most of the young (Greeley *et al.*, 1988).

Ovarian recrudescence in Florida *F. heteroclitus* began at a time [late January/early February (Fig. 1)] of short (LD $\approx$ 11:13) but increasing photoperiod, despite the lowest water temperatures of the year (13–15°C; pers. obs.). The increasing photoperiod and the cold temperature perhaps initiated the recrudescence. Possible environmental correlates of ovarian recrudescence have been explored in fish collected from a Delaware population in late October/December. Ovarian recrudescence of these fish was enhanced by their previous exposure to short days and low temperatures and occurred during long days (LD 13:11 or 15:9) at a warm temperature of 20°C over a period of 6 to 8 weeks (Day and Taylor, 1984). Long days and warm temperature were no longer necessary for recrudescence in females collected in March, and could not sustain reproductive activities of fish collected in late June beyond the normal breeding season. However, when females from the Florida habitat were collected in July and August and from October to February, they were maintained in reproductive condition beyond their breeding season under long photoperiod (LD 14:10) and warm temperature (25°C) without preconditioning (Lin *et al.*, 1989). These females retained high levels of gonadotropin activities in their pituitaries and carried gonadotropin-responsive follicles outside of the breeding season. An annual variation in seasonality in *F. heteroclitus* may dictate its variable ovarian responses to laboratory regimens of photoperiod and temperature (Day and Taylor, 1984; Taylor, 1986).

Nevertheless, the long breeding season in the field, punctuated by lunar and semilunar spawning cycles, collapsed at a time [late September (Fig. 1)] when temperature was still warm (28–30°C) and a uniform photoperiod (LD $\approx$ 12:12) had begun to decline. This collapse was probably not linked directly to a lack of pituitary gonadotropin content (as measured by a homologous

bioassay), which does not collapse in late September but only declines slowly later (Lin *et al.*, 1987). Instead, the steep decrease in vitellogenic and maturing follicles may have been due to a sudden termination of gonadotropin release, which may be linked to starvation. For example, previous studies on *F. heteroclitus* have shown the loss of follicular competence for maturation in starved females, an effect that can be reversed by either feeding or injection of gonadotropin [Wallace and Selman, 1978, 1980; see Holland and Dumont (1975) for similar effects of starvation and gonadotropin on vitellogenesis in *Xenopus laevis*]. The collapse of ovarian activities at the end of summer might, therefore, have been a result of seasonal food shortage (Kneib, 1986; Taylor, 1986; Lin *et al.*, 1989). The decline in size of the largest maturing follicles through the breeding season (Fig. 2) perhaps hinted at a decrease in resources for follicular growth. For *F. grandis*, fat storage (an indication of food availability) has also been linked to reproductive potential (MacGregor *et al.*, 1983). However, because semilunar spawning of *F. heteroclitus* kept in the laboratory and fed also collapsed after five to eight cycles (Figs. 5, 6), the correlation of starvation to the cessation of ovarian activities of wild fish in September is not conclusive. Alternatively, this cessation of reproductive activities can be explained as an expression of an endogenous seasonality progression.

Whether the seasonal transition in the annual cycle directly reflects environmental changes or expresses an underlying endogenous cycle, the transition was interrupted when the fish were collected and moved to the laboratory. During collection and early adaptation in the laboratory, fish usually stop eating and undergo some degree of ovarian regression (Wallace and Selman, 1978, 1980). Regardless of the season (January, April, August, or late September), laboratory fish nevertheless spawned within a few weeks of collection, and all exhibited only semilunar cycles instead of the lunar and semilunar cycles observed in wild fish (Fig. 1). The laboratory semilunar spawning then usually persisted for five to eight regular cycles and generally ended with a short irregular period leading to reproductive regression. The dissimilarity between wild and laboratory fish and the similarity among laboratory groups may suggest that the common stress fish experience during collection may have set their physiological state conducive for semilunar spawning.

Semilunar cycles

Throughout the breeding season, spawning activities of wild northeastern Florida *F. heteroclitus* were supported by successive development cycles of vitellogenic and maturing follicles (Figs. 1, 3). Cyclic ovarian activity thus appears restricted to gonadotropin-sensitive processes, *i.e.*, vitellogenesis and maturation (Wallace and Selman, 1981a). The recruitment of cortical alveolus-stage and early vitellogenic follicles into fully vitellogenic follicles began after each spawning period associated with full/new moon (Fig. 3). Vitellogenic follicles then grew to a diameter of 1.3–1.4 mm, entered maturation (when oocytes hydrate and become eggs), and eventually released their eggs for the next spawning period two weeks later. This organismal synchronization is indeed scheduled by an endogenously timed process as indicated by the laboratory results. The seven spawning groups observed in laboratory aquaria of different sizes exhibited free-running cycles with periods and phases that varied compared to constant concurrent tidal and moonlight cycles (Figs. 5, 6; Table I). The same result has been found for *F. grandis* (Hsiao and Meier, 1992). The possibility of subtle tidal/lunar factors that sustain corresponding activity cycles in laboratory animals (Brown, 1983) thus does not appear to be a serious consideration, either for *F. grandis* or *F. heteroclitus*.

An endogenous timer establishes an organizational basis for cyclic activities (see Introduction) and is generally temperature-compensated, as demonstrated for *F. grandis* (Hsiao and Meier, 1992), to protect its schedule from being destabilized by temperature fluctuations. Although temperature-compensation for the endogenous timer in *F. heteroclitus* has not been tested, its existence would be necessary under natural conditions. The Florida *F. heteroclitus* pace their spawning cycles in synchrony with constant new/full moons and spring tides throughout a temperature range from lows of 13–15°C in the late winter to highs of 28–30°C in the summer. The enhanced formation of distinct follicle clutches during the summer can be interpreted as demonstrating temperature-sensitive follicle growth (Wallace and Selman, 1978) confined by a temperature-compensated format for semilunar spawning. As temperature increases, each follicle clutch proceeds through maturation (and probably vitellogenesis) more rapidly and appears as a narrower and thus distinct band in its size-frequency chart (Fig. 2).

The environmental cues that entrain the endogenous lunar and semilunar cycles of the cyprinodonts are unknown. Because spring tides coincide with the new and full moons on the Atlantic coast, our field data do not have the resolution to discriminate between the involvement of tide and moonlight in spawning entrainment of *F. heteroclitus*. However, a recent laboratory study reveals an entrainment effect of moonlight on this fish (Taylor, 1991). The involvement of a semilunar high-tide cycle on the reproductive cycle of *F. grandis* living along the Gulf coast has also been shown. Spawning of this species follows the local tidal cycle instead of the moonlight cycle (Greeley and MacGregor, 1983; Hsiao and Meier, 1989). Along the Gulf, tidal changes reflect the declinational cycle of

the moon (27.3 days) rather than the synodic cycle of moonlight (29.6 days).

Tidal spawning cycles

Within the breeding season, in addition to lunar and semilunar spawning cycles, our frequent sampling over two-day periods during spring tides revealed a step-by-step ovarian preparation for tidal spawning. Counts of ovulated eggs in ovaries fluctuated dramatically with the daily ebb and flow of the tides. Follicles matured and ovulated in preparation for a spawning selectively directed at the higher tide of the two daily semidiurnal high tides, regardless of the daily light-dark rhythms (Fig. 4). This finding is unique compared to the tidal spawning described for *F. grandis* (Greeley and MacGregor, 1983) and *F. similis* (Greeley *et al.*, 1986), which was based on simple presence of eggs at nonspecific high tides. Specific selection of the higher daily tide obviously maximizes the use of high marsh surface as a spawning ground and minimizes egg loss by subsequent tidal flushing. If this highly specific temporal alignment could be studied in the laboratory, it would surely be a fertile research ground for studies of biological timing and its entrainment. Such a possibility was indirectly revealed in a case where locomotor activity of *F. grandis* was recorded for nine days (MacFarlane and Livingston, 1983). During this period, the differential between the activity rhythm and the light regimen of LD 12:12 produced a gradual delay of the daily peak of locomotor activity (which can be associated with spawning) from the offset to the onset of the light cycle. For directly detecting the daily change of spawning time in the laboratory, however, a monitor must be developed to count eggs at least once an hour.

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